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TGF-β-receptor-mediated signaling

Rik Derynck

Transforming growth factor β (TGF-β) and its many relatives are thought to play key roles in the control of cell proliferation and differentiation. In particular, the ability of TGF-β to induce growth arrest in epithelial cells has drawn considerable attention. The recent cloning of TGF-β receptors, which are considered to be prototypes of a new class of cell-surface receptors, has provided a first insight into how TGF-β signaling induces a variety of intracellular changes. Furthermore, recent advances in the characterization of the cell-cycle machinery have stimulated studies aimed at understanding how TGF-β signaling leads to growth arrest in the late G1 phase of the cell cycle.

MORE THAN a decade ago, transforming growth factor β (TGF-β) was discovered as a factor that induced phenotypic transformation of some fibroblast cell lines. Since then, it has been shown to have a variety of biological activities that affect cell proliferation and differentiation in many cell types. TGF-β stimulates the proliferation of various mesenchymal cell types, but acts as a growth inhibitor of many other cell types, including epithelial cells. Furthermore, it induces the synthesis of extracellular-matrix proteins, modulates the expression of matrix proteases and protease inhibitors, increases integrin expression and thus enhances cell adhesion. TGF-β also affects mesenchymal differentiation and is a very potent chemotactic agent for several cell types, especially monocytes and fibroblasts (for reviews see Refs 1, 2).

Since the initial cDNA cloning of TGF-β, a large superfamily of secreted factors that are structurally related to TGF-β has been discovered. In addition to the three TGF-β isoforms (TGF-β1, -β2 and -β3, each encoded by a separate gene)[3], the TGF-β superfamily comprises the activins and bone morphogenetic proteins (BMPs), as well as many other secreted factors that are all thought to play major roles in differentiation and tissue morphogenesis[4]. The importance of this family is suggested by the structural conservation from *Drosophila* and *Caenorhabditis elegans* to humans. Because the TGF-β-related factors have similar biological activities, TGF-β is considered to be the prototype for this superfamily. Thus, an understanding of the receptor-mediated signaling pathways of TGF-β should provide insight into the mechanisms not only of growth arrest, but also of the activities of all these factors.

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Different types of TGF-β receptors

TGF-β and activin receptors were initially characterized by cell-surface crosslinking experiments using ^{125}I-TGF-β or ^{125}I-activin. Most cells contain two types of receptors, designated type I (65–70 kDa) and type II (85–110 kDa), based on the molecular weight of the crosslinked complex. Many cells also contain a larger type III receptor for TGF-β, which migrates as a diffuse band owing to its proteoglycan nature. Although crosslinking experiments have identified additional cell-surface proteins that bind TGF-β (named type IV through type IX receptors)[5], most attention has focused on types I, II and III, which have now been cloned.

The cDNA sequence of the type IIA activin receptor[6], the first cloned receptor for a TGF-β-related factor, provided insight into the signaling mechanisms of this family of factors. This receptor was identified as a transmembrane serine/threonine (Ser/Thr) kinase with structural homology to the *C. elegans* daf-1 protein, a predicted receptor. Since then, cDNA cloning of various type II and type I receptors for TGF-β, activin, BMP-2 and BMP-4, which are all transmembrane Ser/Thr kinases, has led to the assumption that all TGF-β-related factors signal through type II and type I receptors, which belong to a large family of Ser/Thr kinase receptors[7]. Thus, the receptor signaling of TGF-β is likely to serve as a paradigm for the entire family of ligands and receptors.

The type III receptor. The type III receptor, also called betaglycan, is a transmembrane proteoglycan with a short cytoplasmic domain[8,9]. Its only homology described so far is with endoglin, a transmembrane glycoprotein that binds TGF-β1 and TGF-β3, but not TGF-β2 (Refs 8, 9). The initial suggestion that TGF-β signaling was mediated through the type III receptor has not been sustained, which is consistent with the lack

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of enzymatic motifs in its cytoplasmic domain. Thus, this receptor is not required for biological activity of TGF-β. However, the cytoplasmic domain has a phosphorylation site for protein kinase C, the relevance of which is unknown[8,9]. The type III receptor may be able to interact with the type II receptor (see below), but does not require the type II receptor for TGF-β binding[10,11].

The type II receptor. The type II and type I receptors mediate TGF-β signaling. They are both transmembrane Ser/Thr kinases with a short extracellular domain and a long cytoplasmic region that consists largely of a kinase domain. The large number of cysteines in the extracellular domain and a cluster of three cysteines just upstream of the transmembrane region are characteristic of all kinase receptors for TGF-β superfamily members[7] (Fig. 1).

The one cloned type II TGF-β receptor[12] has a coincident expression with TGF-β1 during development and displays a high affinity for TGF-β1 and TGF-β3 but not TGF-β2. These data suggest that this is probably the major receptor for TGF-β1 and possibly TGF-β3 (Ref. 13). Although TGF-β2 can bind this type II receptor in the presence of the type III receptor[10,11], another type II receptor may exist that has a high affinity for TGF-β2 (Ref. 13). The kinase domain of the type II TGF-β receptor is flanked by a carboxy-terminal extension and a spacer segment between the transmembrane and kinase sequences[12]. The lack of sequence similarity in these segments with the corresponding regions in other members of this receptor family suggests that they may be involved in conferring signaling specificity. Its predicted Ser/Thr kinase activity has been confirmed *in vitro*[6,12] and *in vivo*[14]. However, the TGF-β type II receptor may autophosphorylate not only on serine and threonine, but also on tyrosine, as has been suggested for the activin type II receptor, suggesting dual specificity of the kinase activity.

The type I receptors. In contrast to the single type II TGF-β receptor and the two activin type II receptors, several cloned receptors have been proposed as type I receptors for TGF-β or activin[15,16], which is consistent with the crosslinking patterns of this receptor, which often reveal heterogeneity both within one cell type and between cell types. All are transmembrane Ser/Thr kinase receptors and are structurally closely related, thus representing a distinct subfamily of type I receptors[7]. Several sequence features distinguish them from type II receptors. Their cytoplasmic domains are shorter and contain, besides the characteristic kinase subdomains, a carboxy-terminal extension of only a few amino acids. In addition, the spacer regions between the transmembrane and kinase domains contain a conserved SGSGSGLP motif absent in the type II receptors. Similarly to the type II receptors, they autophosphorylate *in vitro* on serine and threonine, but may also have dual substrate specificity. Their extracellular domains are generally shorter than the corresponding domains in the type II activin or TGF-β receptors and contain, besides the cysteine cluster immediately upstream from the transmembrane region, a distinct spacing of five cysteines, conserved in all type I receptors (Fig. 1).

The binding properties of the type I activin and TGF-β receptors, assessed by crosslinking analysis of transfected receptors, are distinct from those of the type II receptors. Whereas the type II receptors bind their ligand independently[12], binding of TGF-β or activin to the type I receptors requires coexpression with type II TGF-β or activin receptors[16,17]. Several type I receptors have been shown to bind TGF-β or activin in this way, albeit with different efficiencies[16,17]. Thus, the ligand specificity and affinity are conferred by the type II receptor, probably through the physical interaction between the two receptor types (see below). As a result, mutant Mv1Lu cells expressing intact type I receptors, but lacking functional (TGF-β-binding) type II receptors, lack TGF-β binding to both receptor types. Furthermore, transfection of a functional type II receptor into these same cells rescues ligand binding to both receptor types[18]. However, based on ^{125}I-TGF-β crosslinking data, many cell lines seem to lack type II receptors, yet have ligand-binding type I receptors. This may be due to a decreased TGF-β crosslinking efficiency to the type II receptor as a consequence of ligand binding to the type I receptor[15]. Alternatively, as yet unidentified type I receptors that do not require coexpression of type II receptors for ligand binding, or other receptors with which the type I receptors can interact, may exist.

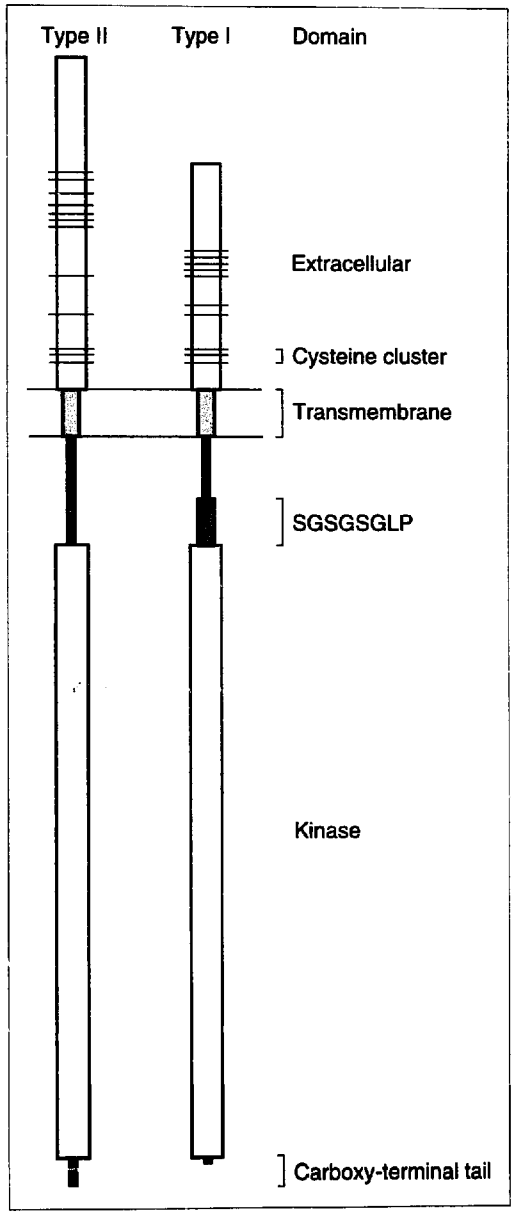

Figure 1
Schematic representation of the type II transforming growth factor β (TGF-β) receptor and a type I receptor. The horizontal lines in the extracellular domains mark the cysteines. The conserved SGSGSGLP sequence characteristic of type I receptors precedes the kinase domain. The cytoplasmic extension following the kinase domain of the type I receptor is only a few amino acids long. The type II receptor is more heavily *N*-glycosylated than the type I receptor.

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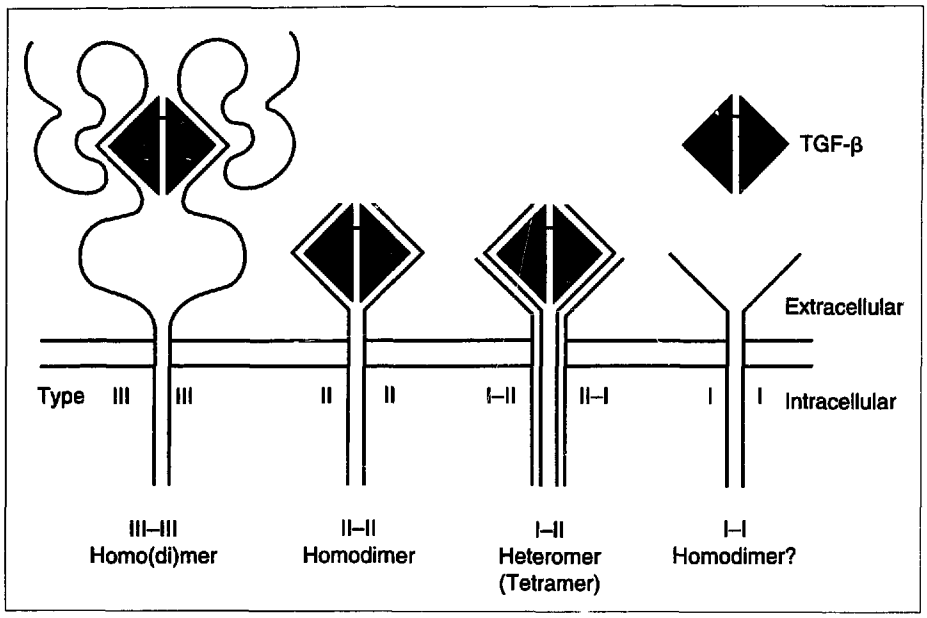

Figure 2

Schematic model of possible homomeric and heteromeric interactions between the different transforming growth factor β (TGF-β) receptor types. Possible interactions between the type III and type II receptors are not shown. The type III receptor is a transmembrane proteoglycan that undergoes homo(di)merization, in both the absence and presence of TGF-β. The type II receptor is also a homodimer, in both the presence and absence of TGF-β. TGF-β does not bind to the type I receptor in the absence of the type II receptor. Finally, the heteromeric complex between type I and type II receptors is shown as a tetramer.

Although the ligand binding and crosslinking experiments suggest heterogeneity among the TGF-β and activin type I receptors, it is as yet unclear whether all these receptors mediate the biological activities of both ligands. Unfortunately, only one set of receptor-negative mutants (derived from a single epithelial cell line, Mv1Lu) exists, which greatly limits a thorough evaluation of their ability to mediate TGF-β responses. Only one type I receptor, named ALK-5, R4 or TβRI, has been shown to confer TGF-β responsiveness in these cells[16,19–21]. However, the type I activin/TGF-β receptor, named Tsk7L, R1, ALK-2, SKR-1 or ActR-I, may mediate TGF-β responses in another epithelial cell line that lacks ALK-5 expression[22]. The diversity of type I receptors for TGF-β (or activin) may reflect a partial physiological redundancy and the ability of these receptors to mediate different spectra of TGF-β responses. Also, some preferential use of type I receptors may occur, depending on the cell type. Whereas ALK-5 may be a predominant type I TGF-β receptor in many cell types, a diversity of type I TGF-β receptors would be consistent with the cell-type-dependent heterogeneity in type I receptor crosslinking patterns and the structural similarity of these receptors. The complexity of type I receptors may also explain in part the diversity of TGF-β responses among different cell types. A similar diversity of combinatorial interactions has been observed in other receptor systems as well. As an example, a common gp130 receptor component and a common β-chain interact with two distinct sets of cytokine receptor complexes and mediate the signaling of these different cytokines[23]. A preference for which type I receptors are used, depending on the cell line, would be analogous to the cell-type-dependent preferred usage of the receptor-associated kinases JAK1, JAK2 and TYK2 as signaling mediators for various cytokine receptors[24].

Homomeric and heteromeric receptor interactions

To gain insight into how TGF-β exerts its multiple activities, it is necessary to understand the physical and physiological interactions between the different receptor types (Fig. 2). The required co-expression of the type II and type I receptors for TGF-β responsiveness and the co-immunoprecipitation of both receptor types following crosslinking of ^{125}I-TGF-β (Refs 16–18) have led to the proposal that the functional TGF-β receptor is a heteromeric complex of type II and type I receptors[18]. Although these results demonstrated the ability of both receptor types to form a heteromeric complex, they did not reveal the stoichiometry of this complex, the fraction of the receptors that is engaged in heteromerization, or whether heteromerization is required for TGF-β signaling. In addition, the type II receptors have recently been shown to form stable homomeric complexes, presumably dimers, in both the presence and the absence of TGF-β (Refs 25–27). This constitutive homomeric complex formation may resemble the insulin and insulin-like growth factor (IGF-1) receptors, which also form homodimers in both the presence and the absence of their ligand[28]. The homodimerization of type II receptors, even in the presence of type I receptors, and the existence of type I receptor homodimers make it likely that the heteromeric complex is a tetramer consisting of the constitutive type II homodimer and two type I receptors[27]. The type III receptors also form homo-oligomers or homodimers independently of ligand binding[25]. Co-immunoprecipitation experiments have demonstrated a physical interaction of type III with type II receptors following binding and crosslinking to ^{125}I-TGF-β. This led to the proposal that TGF-β binding to the type III receptor induces association with the type II receptor[10,11]. More recent studies on the total population of type III receptors failed to show an association between type III and type II receptors, suggesting that only a minimal fraction of the receptors engages in type III–type II receptor interactions and/or that these interactions are very transient[25]. The increased affinity of the type II receptor for TGF-β when coexpressed with the type III receptor suggests that the latter may be involved in ligand presentation to the type II receptor. This may be especially relevant in the case of TGF-β2, since the type III receptor greatly enhances the otherwise minimal affinity for TGF-β2 (Refs 10, 11).

By analogy with receptor tyrosine kinases, it might be expected that TGF-β induces receptor clustering and auto-/transphosphorylation; however, the

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current results do not provide conclusive evidence that this is the case. Ligand binding is not required for homodimerization of the type III and type II receptors[25,26], but may induce a transient association of these two receptor types. With respect to type-I–type-II receptor heteromerization, it has been proposed that ligand induces the interaction between both receptor types[14]. However, they already associate in the absence of ligand and have an inherent affinity for each other. Thus, it is likely that, similarly to receptor tyrosine kinase receptors[29], the interaction between the two receptor types pre-exists but is stabilized by ligand occupation. The phosphorylation-dependent direct interaction of the type I and type II receptor cytoplasmic domains suggests that phosphorylation of the cytoplasmic domains plays a role in the stabilization of the heteromeric complex. Analysis of the *in vivo* phosphorylation of transfected receptors shows that, similarly to type II activin receptors[30], the type II TGF-β receptors are phosphorylated on multiple serines and threonines in both the absence and the presence of TGF-β (Ref. 14). Many of these sites are also phosphorylated when a kinase-defective mutant of the type II receptor is transfected, suggesting that they are phosphorylated by other kinases, which is consistent with the presence of potential protein kinase C and casein kinase II phosphorylation sites in the cytoplasmic domain. However, the type II receptor kinase activity is required for the autophosphorylation of several other sites in the cytoplasmic domain. The heteromeric interaction of type II and type I receptors suggests the attractive possibility that the two receptor types transphosphorylate each other's cytoplasmic domains, which may play a role in the stabilization of the receptor complex. Thus, in a heterotetrameric complex, not only autophosphorylation, but also transphosphorylation of both receptor types could be envisaged, leading to receptor activation and interaction with signaling targets. Accordingly, it has been shown that the type II receptor phosphorylates the type I receptor in the heteromeric complex[14].

Two models have been proposed for the contribution of the type I and type II receptors to the TGF-β activities. One model proposes that signaling of both type I and type II receptors is required for the biological activities and that the absence or inactivation of one or another cytoplasmic domain abolishes all biological activities[7]. This model is based on experiments using transfected receptors and their kinase-defective mutants in Mv1Lu cell mutants lacking functional receptors. The other model proposes two distinct receptor-associated signaling pathways[31]. Thus, the growth inhibition by TGF-β requires signaling through the type II receptor, presumably in combination with the type I receptor. However, the second pathway is mediated through the type I receptor without signaling through the type II receptor – as long as the extracellular domain of the type II receptor provides ligand interaction with the type I receptor – and induces a defined set of TGF-β activities. This second model is based on the observation that transfection of a truncated type II receptor lacking a cytoplasmic domain into Mv1Lu cells inhibits the antiproliferative effect of TGF-β in a dominant-negative way, yet does not inhibit TGF-β induction of several other responses[31]. The latter model is consistent with several previous observations in other cell systems. Indeed, complementation analysis of cell hybrids revealed that acquisition of the growth inhibitory response correlated with type II receptor expression[32]. Furthermore, several cell lines lack a functional type II receptor and are not growth inhibited by TGF-β, yet respond to TGF-β with increased synthesis of extracellular-matrix molecules (see, for example, Ref. 33). Finally, various tumor cell lines are not growth inhibited by TGF-β, yet respond by increased extracellular matrix synthesis, thus illustrating the uncoupling of the antiproliferative effect of TGF-β from its effects on extracellular matrix production. A similar phenotype is obtained following inactivation of the retinoblastoma protein (RB) by oncoproteins such as adenovirus E1A or SV40 large T antigen[34]. This selective loss of the antiproliferative effect of TGF-β has been considered as an early step in the malignant progression of a tumor cell. Finally, in smooth-muscle cells and osteoblasts, the ligand binding and availability of type I and type II receptors are independently regulated, and this correlates with alterations in the spectra of responses to TGF-β. Further characterization of the receptor interactions and kinase activities, as well as their targets, will be important to determine how they mediate their biological activities.

TGF-β-induced signaling events

Very little is known about the intracellular events that follow TGF-β treatment. The signaling mechanisms leading to changes in proliferation by TGF-β are likely to be different in cells that are growth stimulated versus those that are growth arrested by TGF-β. In fibroblasts and other mesenchymal cell types, TGF-β induces the expression of endogenous growth factors such as platelet-derived growth factor (PDGF) and connective tissue growth factor, or increases the expression of epidermal growth factor (EGF) receptors. In addition, TGF-β induces the synthesis of several extracellular-matrix proteins and their integrin ligands. Thus, in these cells, TGF-β-induced signaling events that lead to growth stimulation may result indirectly from growth factor activation of receptor tyrosine kinases or altered cell–matrix interactions, rather than directly from TGF-β receptor signaling. Because of space limitations, I will only discuss growth inhibition by TGF-β here.

Most studies of TGF-β-induced growth inhibition have focused on its effect on the cell-cycle machinery, but the upstream events leading to this regulation are largely unknown. However, three important findings have been made in cells that are growth arrested by TGF-β. (1) In several epithelial cell lines, TGF-β appears to activate Ras (Ref. 35), and to increase mitogen-activated protein kinase (MAPK) activity. The speed of this response suggests a close link with receptor activation. This result supports previous suggestions that G proteins may be involved in TGF-β signaling. (2) TGF-β induces protein phosphatase 1 activity in keratinocytes[36]. (3) TGF-β induces phosphorylation of the transcription factor CREBP (cAMP response element binding protein) in Mv1Lu cells, without affecting the protein level of CREBP[37]. How these signaling events are mediated following receptor activation, and whether they play a role in the antiproliferative effect or other activities of TGF-β, remains to be determined.

TGF-β and cell-cycle arrest. TGF-β arrests the cell cycle of epithelial cells when administered during early G1, but with a decreasing efficiency as the cells approach late G1 (Refs 38, 39). Following the G1–S transition, the cells are largely unresponsive to TGF-β and to extracellular mitogenic factors.

RB is a central regulator and target of the cell-cycle machinery, and its

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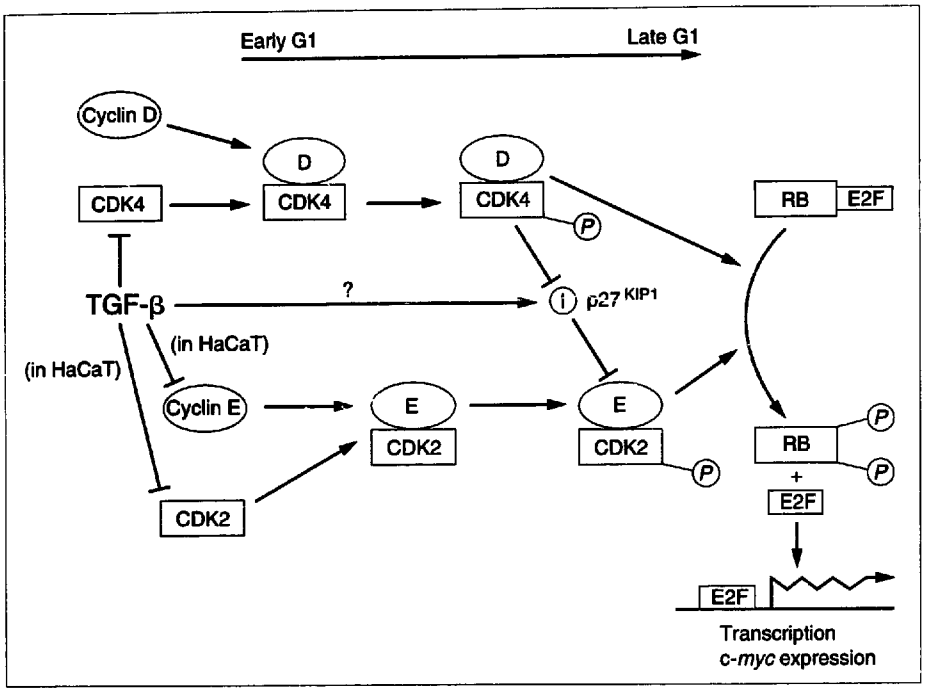

Figure 3

Possible effects of transforming growth factor β (TGF-β) on the components of the cell-cycle machinery leading to growth arrest in late G1 phase. This model is based on the findings discussed in the text and, in part, on a diagram in Ref. 40. The induction of cyclin D synthesis precedes the induction of cyclin E expression during G1 phase. TGF-β inhibits CDK4 synthesis. The induction of cyclin E and CDK2 expression is inhibited in HaCaT cells[39], but not in Mv1Lu cells. This diagram suggests that TGF-β may regulate $p27^{KIP1}$, a postulated inhibitor of cyclin-E–CDK2, but the evidence for a direct connection with TGF-β is rather weak. It is, as yet, unclear how $p15^{INK4B}$, an inhibitor whose expression is upregulated by TGF-β (Ref. 47), fits into this model. Cyclin-D–CDK4 and cyclin-E–CDK2 complexes mediate RB phosphorylation. E2F released from RB following phosphorylation plays an important role in the G1–S transition.

phosphorylation state is cell-cycle dependent. It is dephosphorylated as cells exit mitosis and phosphorylated just before the G1–S transition. In its underphosphorylated form, RB acts as a growth suppressor and prevents G1–S transition, and this activity is linked to its ability to bind the transcription factor E2F and several other transcriptional regulators. Following phosphorylation of RB, E2F is released and activates a defined set of genes required for G1–S transition and DNA replication[40]. Several transforming proteins, such as the SV40 large T antigen, adenovirus E1A and human papillomavirus E7 protein, associate with underphosphorylated RB and thus inactivate its growth-suppressing activity[41]. Growth arrest in late G1 induced by TGF-β or serum starvation is accompanied by the maintenance of RB in its underphosphorylated form, which has led to the suggestion that TGF-β might play a role in determining the phosphorylation state of RB[38]. To understand how TGF-β may affect RB phosphorylation and induce growth arrest, several studies have evaluated the effect of TGF-β on G1 cyclins and the associated cyclin-dependent kinases (CDKs). Among these, cyclin D, which exists as three isoforms encoded by distinct genes, can associate with CDK4, whereas cyclin E forms a complex with CDK2. Both types of complexes are thought to mediate RB phosphorylation before the G1–S transition[40]. Although each of the few cell lines studied had a somewhat different pattern of cyclins and CDKs affected by TGF-β, some important observations have been made.

TGF-β treatment inhibits CDKs. In the epithelial cell lines studied, TGF-β does not affect the moderate and transient increases in cyclin D1 and D2 expression that occur during early G1 phase. Rather, TGF-β acts by inhibiting the concomitant induction of their major partner, CDK4 (Refs 39, 42) and can thus block entry into S phase when added after the increase in cyclin D expression[39]. At a later stage during G1, expression of cyclin E and its partner CDK2 is induced strongly. This induction is nearly abolished by TGF-β in HaCaT cells[39], but not in Mv1Lu epithelial cells[42,43]. However, in Mv1Lu cells, CDK2 is inactive, perhaps because it fails to assemble with cyclin E (possibly as a result of an inhibition of CDK2 phosphorylation)[43] or because it associates with an inhibitor (see below). However, whether this effect is the direct result of TGF-β receptor activation or is a consequence of more general effects of TGF-β-induced growth inhibition is not clear. Finally, TGF-β blocks the induction of cyclin A expression, which normally occurs shortly before the point in G1 at which TGF-β arrests the cell cycle[39]. Constitutive overexpression of CDK4, but not CDK2, in Mv1Lu confers resistance to the antiproliferative effect of TGF-β, thus resulting in phosphorylation of RB in late G1 and entry into S phase even in the presence of TGF-β (Ref. 42). Furthermore, TGF-β-induced downregulation of CDK4 inhibits CDK2 activation in Mv1Lu cells, even though cyclin E and CDK2 expression are not affected[42]. These results emphasize the role of CDK4 in TGF-β-induced growth arrest in late G1 and underscore a functional link between CDK4 and CDK2 activation (Fig. 3).

Several CDK inhibitors that interact with the cyclin–CDK complexes have been identified: p21 inhibits all CDKs studied, with preference for CDK2, and may participate in p53-induced cell-cycle arrest by inhibiting cyclin-E–CDK2 activation[44]; $p27^{KIP1}$ also inhibits activation of cyclin E-CDK2 complexes and can be found associated with these complexes in growth-arrested cells[45]; and p16 inhibits CDK4 activity selectively. One or several of these inhibitors are likely to play a role in TGF-β-mediated growth arrest, especially because of the crucial roles of CDK4 and CDK2 in RB phosphorylation and G1–S transition, but no convincing evidence has as yet been provided for a direct mechanistic link between these CDK inhibitors and TGF-β signaling. By contrast, the expression of a recently identified p16-related CDK inhibitor, $p15^{INK4B}$, has been shown to be upregulated in

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HaCaT cells following TGF-β treatment, suggesting its involvement in TGF-β-induced growth arrest[47]. Further characterization of the cell-cycle machinery and its role in growth arrest in late G1 is required before specific targets of the TGF-β signaling events that lead to growth inhibition will be identified convincingly.

Another protein that may play a role in TGF-β-induced growth arrest is c-Myc, since TGF-β can downregulate c-*myc* expression in some cell lines that are responsive to its antiproliferative effect[41]. In addition, several pieces of evidence suggest that RB function and c-*myc* expression are interconnected. First, TGF-β-induced downregulation of c-*myc* expression is abrogated in cells that express viral transforming proteins, such as adenovirus E1A and HPV E7, that associate with RB and inactivate its function as a growth suppressor[41]. Furthermore, overexpression of c-*myc* can abolish G1 arrest induced by RB[48]. Finally, the c-*myc* promoter has two binding sites for the protein E2F, the activity of which is regulated by phosphorylation of RB[49]. However, further study is required to clarify the role of c-Myc in growth arrest induced by TGF-β, and to evaluate a possible functional connection between cyclin/CDK/RB regulation and c-Myc.

Clearly, we have only just begun to understand how TGF-β and its relatives exert their multiple activities. Further characterization of these new types of receptors and the cell-cycle machinery is required before the receptor-mediated signaling events can be fully elucidated. As a necessary step in the analysis of the signaling cascade, several labs are characterizing proteins that can interact with the TGF-β receptors and could thus be involved in TGF-β-receptor-mediated signaling. Recently, the immunophilin FKBP-12 was shown to interact with type I receptors[50], but the physiological relevance of this interaction in TGF-β signaling remains to be determined. Considering the recent cloning of a number of TGF-β receptors and the complexity of the interactions of the downstream signals, great caution should be exercised before conclusions are drawn about the mechanisms of TGF-β signaling.

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